

# Increased Levels of Macrophage Inflammatory Proteins Result in Resistance to R5-Tropic HIV-1 in a Subset of Elite Controllers

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## ABSTRACT

Elite controllers (ECs) are a rare group of HIV seropositive individuals who are able to control viral replication without antiretroviral therapy. The mechanisms responsible for this phenotype, however, have not been fully elucidated. In this study, we examined CD4<sup>+</sup> T cell resistance to HIV in a cohort of elite controllers and explored transcriptional signatures associated with cellular resistance. We demonstrate that a subgroup of elite controllers possess CD4<sup>+</sup> T cells that are specifically resistant to R5-tropic HIV while remaining fully susceptible to X4-tropic and vesicular stomatitis virus G (VSV-G)-pseudotyped viruses. Transcriptome analysis revealed 17 genes that were differentially regulated in resistant elite controllers relative to healthy controls. Notably, the genes encoding macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ), *CCL3* and *CCL3L1*, were found to be upregulated. The MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES chemokines are natural ligands of CCR5 and are known to interfere with HIV replication. For three elite controllers, we observed increased production of MIP-1 $\alpha$  and/or MIP-1 $\beta$  at the protein level. The supernatant from resistant EC cells contained MIP-1 $\alpha$  and MIP-1 $\beta$  and was sufficient to confer R5-tropic resistance to susceptible CD4<sup>+</sup> T cells. Additionally, this effect was reversed by using inhibitory anti-MIP antibodies. These results suggest that the T cells of these particular elite controllers may be naturally resistant to HIV infection by blocking R5-tropic viral entry.

## IMPORTANCE

HIV is a pandemic health problem, and the majority of seropositive individuals will eventually progress to AIDS unless antiretroviral therapy (ART) is administered. However, rare patients, termed elite controllers, have a natural ability to control HIV infection in the absence of ART, but the mechanisms by which they achieve this phenotype have not been fully explored. This paper identifies one mechanism that may contribute to this natural resistance: some elite controllers have CD4<sup>+</sup> T cells that produce high levels of MIP chemokines, which block R5-tropic HIV entry. This mechanism could potentially be exploited to achieve a therapeutic effect in other HIV-seropositive individuals.

In the absence of antiretroviral therapy (ART), the majority of HIV-seropositive patients have detectable viral loads (VLs) and experience a slow but inevitable decline in the number of CD4<sup>+</sup> T cells, eventually culminating in the development of AIDS. However, in rare individuals (<1% of the HIV-seropositive population), viral replication is suppressed to extremely low or undetectable levels. These patients, termed elite controllers (ECs), typically retain relatively high CD4<sup>+</sup> T cell counts and do not progress to AIDS, even in the absence of ART (1–4). Sequencing of HIV and *in vitro* functional assays suggest that most ECs possess replication-competent virus (5). Long-term nonprogressors (LTNPs), representing ~2 to 15% of HIV-seropositive patients, are a second group of individuals with a protective phenotype, in whom the virus continues to replicate but at a reduced level. In LTNPs, the CD4<sup>+</sup> T cell count decline and progression to AIDS are significantly delayed (6–9). Genome-wide association studies have revealed several alleles within the human leukocyte antigen B/C (HLA-B/C) block that are enriched in both HIV ECs and LTNPs versus individuals with normal HIV disease progression (termed progressors) (10). Notably, B\*57:01, B\*27:05, B14/Cw\*08:02, B\*52, and A\*25 alleles are protective, whereas B\*35 and Cw\*07 alleles confer an elevated risk. However, some ECs lack protective HLA alleles, and other HIV-seropositive patients exhibit viremia and disease progression despite possessing these protective alleles. Collectively, these alleles account for only 20% of the overall vari-

ance in HIV control (10). Hence, additional mechanisms must contribute to the virologic suppression characteristic of ECs. Determining how ECs are able to control HIV replication may expand our knowledge of HIV pathogenesis, reveal novel therapeutic targets, and inform vaccine and eradication efforts.

While the mechanisms of viral containment remain largely unknown, a number of key features have been associated with elite control (1, 11, 12). ECs exhibit T cell responses that are qualitatively different from HIV progressors. CD4<sup>+</sup> T cells from ECs retain their ability to proliferate and produce interleukin-2 (IL-2) in response to HIV (2, 13–15). ECs do not exhibit some of the

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immune changes that are observed in progressors, such as upregulation of cytotoxic-T lymphocyte-associated antigen 4 (CTLA-4) on CD4<sup>+</sup> T cells (16, 17). Interestingly, some ECs have been shown to harbor lower levels of integrated HIV DNA but higher levels of 2-long terminal repeat (2-LTR) circular HIV DNA, suggesting a block at integration post-nuclear entry (18). CD8<sup>+</sup> T cells from ECs also exhibit a more polyfunctional response to HIV, with greater degranulation and release of multiple cytokines, including gamma interferon (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), IL-2, and macrophage inflammatory protein 1 $\beta$  (MIP-1 $\beta$ ) (2, 13, 14, 19–22). Moreover, CD8<sup>+</sup> T cells from ECs exhibit a superior ability to control HIV in cocultured CD4<sup>+</sup> T cells, through cytotoxic activity (23). Because of the complex interplay of HIV with the host immune system, it is likely that some of these features reflect the primary mechanisms of elite control, whereas others reflect downstream effects or events.

Previous studies have yielded conflicting results regarding whether EC CD4<sup>+</sup> T cells are resistant to HIV. Several studies demonstrated that phytohemagglutinin (PHA)-activated CD4<sup>+</sup> T cells from ECs and LTNP are fully susceptible to infection (24–28). Two reports demonstrated that CD3-activated CD4<sup>+</sup> T cells from ECs were resistant to HIV infection in culture (29, 30), while a third study using CD3 activation found comparable HIV infection in CD4<sup>+</sup> T cells from ECs versus progressors (31). Two studies found that unstimulated CD4<sup>+</sup> T cells from ECs and progressors were equally susceptible to HIV infection (32, 33), while a third study found that unstimulated CD4<sup>+</sup> T cells from ECs exhibited a block to viral integration (34). These studies used different activation protocols and various viral strains. Thus, it is hard to ascertain if the conflicting and inconsistent results are a consequence of disparate experimental conditions or dissimilar characteristics of the EC cohorts.

The HIV vectors used in laboratory studies can be engineered to incorporate different HIV envelopes, which vary in their coreceptor usage. CCR5 (R5)-tropic envelopes predominate during the early stage of clinical HIV infection, while CXCR4 (X4)-tropic HIV envelopes may evolve as patients progress to AIDS. Additionally, many laboratory studies use HIV vectors pseudotyped with vesicular stomatitis virus G protein (VSV-G), and recent evidence suggests that the ubiquitously expressed low-density lipid receptor (LDL-R) plays a critical role in its viral entry (35). In this study, we examined EC CD4<sup>+</sup> T cell resistance to different HIV envelopes. We identified a subset of ECs in our cohort who exhibited CD4<sup>+</sup> T cell resistance to R5-tropic HIV but full susceptibility to X4- and VSV-G-enveloped viruses. We investigated transcriptional signatures that were associated with cellular resistance and determined that the MIP-1 $\alpha$  and/or MIP-1 $\beta$  level was elevated in three out of four individuals with the cellular resistance phenotype. These elevated levels of chemokines were necessary and sufficient to confer this phenotype to other cells in a medium transfer experiment.

## MATERIALS AND METHODS

**Peripheral blood mononuclear cell collection and CD4<sup>+</sup> T cell purification.** The Yale Human Investigations Committee and Texas Tech University Health Sciences Center Institutional Review Board approved all studies involving human subjects. Following informed consent, blood was collected from subjects by peripheral venous phlebotomy. Peripheral blood mononuclear cells (PBMCs) were isolated by using Ficoll-Paque Plus reagent (GE Healthcare Life Sciences, Piscataway, NJ), and CD4<sup>+</sup> T

cells were purified via magnetic enrichment using CD4 microbeads (Miltenyi Biotec, San Diego, CA). Purity was judged to be between 85% and 97% based upon flow cytometric analysis (the remaining cells were predominantly CD4-low monocytes with <1% contaminating CD8<sup>+</sup> T cells and <0.1% contaminating NK cells). Cells were frozen in 10% dimethyl sulfoxide (DMSO)–90% fetal bovine serum (FBS) and stored until use.

**CD4<sup>+</sup> T cell activation, single-cycle HIV infection assay, and conditioned medium transfer.** CD4<sup>+</sup> T cell aliquots were thawed and then activated by using plates coated with 1  $\mu$ g/ml anti-CD3 (clone OKT3; eBioscience, San Diego, CA) in the presence of 2  $\mu$ g/ml soluble anti-CD28 (clone 28.2; eBioscience) and 100 IU/ml IL-2 for 3 days. They were then infected with a replication-defective HIV vector encoding a fluorescent reporter gene (enhanced yellow fluorescent protein [eYFP] or enhanced green fluorescent protein [eGFP]) and individually pseudotyped with various R5, X4, and VSV-G envelopes, prepared as previously described (36). Three days later, flow cytometry was performed with a FACSCalibur or FACSCanto instrument (Becton Dickinson, Franklin Lakes, NJ) to quantify green fluorescent protein-positive (GFP<sup>+</sup>)/YFP<sup>+</sup> HIV-infected cells. For the R5-tropic viruses that exhibited a low level of infection, a large number of cells ( $5 \times 10^4$  to  $2 \times 10^5$ ) were analyzed, and gating was set on mock-infected cells such that at most 1 out of  $10^5$  cells was scored positive. Thus, with an infection rate of 1%, the dynamic range of the flow cytometric assay was  $\sim 1,000$ . In some experiments, cells were incubated in supernatants harvested from CD4<sup>+</sup> T cells activated 3 days earlier or supernatants from 293T cells transfected with the following plasmids: pCCL3L1, encoding MIP-1 $\alpha$  (Origene, Rockville, MD); pCCL4, encoding MIP-1 $\beta$  (to generate this plasmid, the *CCL4* coding sequence was amplified from human cDNA and then ligated into plasmid pcDNA3/1+CAT); and pK B-arrestin, encoding  $\beta$ -arrestin (a gift from Bryan Cullen, Duke University). Treatment was performed in the absence or presence of 70  $\mu$ g/ml polyclonal goat IgG neutralizing antibodies against MIP-1 $\alpha$  and/or MIP-1 $\beta$  or the isotype control (R&D Systems, Minneapolis, MN), followed by the addition of HIV-pseudotyped particles 20 min later.

**Quantification of T cell purification, activation, and CCR5 levels.** To assess CD4<sup>+</sup> T cell purity, activation status, and CCR5 levels, samples were stained with fluorescently labeled antibodies against human CD4-allophycocyanin (APC) (RPAT4), CD3-peridinin chlorophyll protein (PerCP)-cyanine 5.5 (clone OKT3), CD8a-phycoerythrin (PE) (HIT8a), and CD14-fluorescein isothiocyanate (FITC) (61D3) or CCR5-PE (NP-6G4), CD69-FITC (FN50), CD25-PerCP (BC96), CD337(NKp30)-PE (AF29-4D12), and CD56-FITC (TULY56) (all from eBioscience). Cells were stained in 2% FBS in phosphate-buffered saline (PBS) on ice for 30 min, washed in the same buffer, and then fixed in intracellular (IC) fixation buffer (eBioscience) on ice for 10 min. Cells were washed and resuspended in 2% FBS in PBS, and flow cytometry analysis was then performed with a FACSCalibur or FACSCanto instrument (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR).

**RNA sequencing (RNA-Seq).** Total RNA was isolated from CD4<sup>+</sup> T cells of ECs and healthy donors by using the RNeasy minikit (Qiagen, Germantown, MD). Isolated RNA was analyzed at the Yale Genome Analysis Center, where cDNA sequencing library preparation and Illumina single-end sequencing were performed. DNA sequence data generated were stored in FASTQ format, and quality control was performed by using FastQC version 0.10.1 (37). Low-quality reads ( $Q < 20$ ) were removed prior to alignment. Quality-filtered reads were aligned to sequences of the human genome (hg19) downloaded from Illumina's iGenome resource (Illumina, San Diego, CA), using TopHat version 2.0.5 (38), as previously described (39). Successfully aligned reads were then further analyzed by using Cuffdiff in order to allow the estimation of differential gene expression using functions of the R package “cummeRbund” (40).

**Whole-exome sequencing.** Genomic DNA was isolated from 10 ml whole blood by using the QIAamp DNA blood maxikit (Qiagen). Targeted capture was performed by using the NimbleGen 2.1 exome reagent

followed by sequencing on the Illumina Genome Analyzer IIx platform. Sequences were aligned to NCBI build 36 of the human genome by using the ELAND program (Illumina) for single nucleotide variation detection and by BWA software for indel detection. Reads aligned out of the targeted intervals and pairs of reads sharing the same start and end positions were regarded as PCR duplicates and discarded. Single-nucleotide variants (SNVs) and indels were called from the remaining reads by using Samtools software and annotated by using an in-house annotation pipeline. dbSNP (build 135) and 1000 Genomes (May 2011 release) were used to test for novelty of variations.

**qPCR and reverse transcription-PCR (RT-PCR).** Genomic DNA and total RNA were isolated as described above. cDNA was synthesized with the Promega reverse transcription system using random primers (Promega, Madison, WI). The *CCR5* genotype ( $\Delta 32$  versus WT) was determined by quantitative PCR (qPCR) with primers CCR5-583F (5'-ACCTCGAGCTCTCATTTTCC-3') and CCR5-664R (5'-CCAGCCCCAAGATGACTATC-3'), as reported previously (41). To quantify *CCR5*, *STAT-1*, *C/EBP $\alpha$* , and *RUNX1* mRNA levels, relative to the *EF1 $\alpha$*  reference gene, qPCR was performed by using the iQ SYBR green Supermix reagent (Bio-Rad), with primers CCR5-F1 (5'-CAAAAAGAAGGTCTTCATTACACC-3'), CCR5-R1 (5'-CCTGTGCCTCTTCTTCATTTCG-3'), hSTAT1F (5'-TGGGTTTGACAAAGGTTCTT-3'), hSTAT-1R (5'-TATGCAGTGCACCGAAAG-3') (42), CEBP $\alpha$ F (5'-CGGTGGACAAGAACAGCAAC-3'), CEBP $\alpha$ R (5'-CGGAATCTCCTAGTCTCTGGC-3') (43), RUNX1F (5'-ACTCGGCTGAGCTGAGAAATG-3'), RUNX1R (5'-GACTTGCGGTGGGTTGTG-3'), EF1 $\alpha$ F (5'-TCTGGTTGGAATGGTGACAACATGC), and EF1 $\alpha$ R (AGAGCTCACTCAAAGCTTCATGG-3') (44). To measure the copy number of *CCL3-like* (*CCL3L*) and *CCL4-like* (*CCL4L*) genes, qPCR was performed on genomic DNA by using the TaqMan no-Amp Erase UNG reagent (Applied Biosystems, Life Technologies, Grand Island, NY), with primers CCL3L1GF (5'-TCTCCACAGCTTCTTAACCAGA-3'), CCL3L1GR (5'-CTGGACCCACTCCTCACTGG-3'), CCL4L1GF (5'-CCCTGGCCTTTCCTTCAG-3'), CCL4L1GR (5'-AAGCTTCTCGCGGTGTAAG-3'), bglobinGF (5'-GGCAACCCTAAGGTGAAGC-3'), and bglobinGR (5'-GGTGAGCCAGGCCATCACTA-3') and the TaqMan probes CCL3L1P (5'-6-carboxyfluorescein [FAM]-AGGCCGCAAGTCTGTGCTGA-6-carboxytetramethylrhodamine [TAMRA]-3'), CCL4L1P (5'-FAM-CAGACCTCCACCGCCTGCT-TAMRA-3'), and bglobinP (5'-VIC-CATGGCAAGAAAGTGCTCGGTGCCT-TAMRA-3') (Applied Biosystems) (45). A total of 6.6 ng DNA template was used for this assay. A previous report indicated that the CEM cell line contains 2 copies of both the *CCL3L1* and *CCL4L1* genes (46); this cell line was used as a reference to determine the copy number for each EC genomic DNA sample relative to the  $\beta$ -globin gene. However, it has recently emerged that there is a complex architecture within the locus encoding the *CCL3-like* and *CCL4-like* genes, including multiple full-length and partial-length genes with a high degree of homology that may be mutually amplified by these primers (47). Hence, results are reported as the relative copy number ( $n$ ) of *CCL3-like* and *CCL4-like* genes for each individual, normalized to values for the CEM cell line ( $n = 2$ ), calculated by using the means from three experimental replicates. Values obtained are reported to at least two significant digits and not rounded to whole integers.

**HLA typing.** The preliminary HLA-B type was determined via sequencing (Yale Department of Surgery Histocompatibility Laboratory Service); B\*57 alleles were subsequently resolved with the HLA B\*57 SSP genotyping kit (Olerup, Stockholm, Sweden).

**ELISA.** MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES instant enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) were used to measure chemokine levels in culture supernatants, according to the manufacturer's instructions.

**Graphs and statistics.** Graphs show either representative results or pooled results from at least two experimental repeats, as indicated in the figure legends. For each assay, a normality test was performed on the healthy control data, and a parametric or nonparametric test was then

performed as appropriate, followed by multiplicity-adjusted posttests for selected pairs. The statistical tests used for each data set are reported in the figure legends. The  $P$  values for pairwise tests, or multiplicity-adjusted posttests of selected pairs, are reported on the graphs. For the transcriptome analysis,  $P$  values were calculated by Cufflinks from the difference in the distributions of observed log fold changes (i.e., Student's  $t$  test on the  $\log_2$ -fold change).

## RESULTS

**Recruitment of a cohort of HIV ECs.** We identified 11 ECs from local clinics in Southern Connecticut, using inclusion criteria of HIV seropositivity, viral loads (VLs) of <400 copies/ml (before 2008) or <48 or <20 copies/ml (after 2008) on at least 2 occasions separated by a minimum of 6 months in the absence of ART, and no AIDS-defining illnesses. Exclusion criteria included clear virologic failure (e.g., progressively increasing VLs) or any contraindication to peripheral phlebotomy. Table 1 shows the clinical characteristics of this cohort. The cohort was heterogeneous in nature and included both genders and a mixture of races and ethnicities. EC-1 met the study criteria at the time of enrollment but subsequently had an elevated plasma viral load, accompanied by a reduced CD4<sup>+</sup> T cell count, and began treatment with efavirenz, emtricitabine, and tenofovir in 2013. EC-3 was prescribed efavirenz, emtricitabine, and tenofovir in 2010 due to a decreasing CD4<sup>+</sup> T cell count that occurred during chemotherapy for rectal cancer, although his viral load remained undetectable. The nine other patients remained ART naive. HLA typing demonstrated that 7/11 patients in the cohort possess a protective B\*57 allele (EC-2, EC-4, EC-5, EC7, EC-8, EC-9, and EC-11).

**A subset of ECs exhibits a novel phenotype of CD4<sup>+</sup> T cell resistance to R5-tropic single-cycle HIVs.** In order to examine T cell resistance to HIV infection in our cohort, we obtained blood samples from recruited patients, purified CD4<sup>+</sup> T cells, and performed a single-cycle HIV infection assay. A *post hoc* analysis revealed a subset of four ECs (EC-1, -2, -3, and -11) whose CD4<sup>+</sup> T cells exhibited relative resistance to YU2-pseudotyped virus, in comparison to the remaining ECs (EC-4 through -10) and healthy controls (Fig. 1A). In contrast, these T cells were fully susceptible to VSV-G-pseudotyped particles (Fig. 1B). For EC-2 and EC-11, we confirmed resistance by using a different HIV backbone and a dose range of YU2 Env-pseudotyped virus (EC-1 and EC-3 were excluded from this analysis because of limited sample availability) (Fig. 1C). The T cells remained fully susceptible to VSV-G-pseudotyped virus under similar conditions (Fig. 1D).

The contrasting results between YU2- and VSV-G-pseudotyped virus led us to question whether the resistance phenotype extended to other R5- and X4-tropic envelopes. We exposed CD4<sup>+</sup> T cells to a panel of replication-defective viruses pseudotyped with different envelopes. CD4<sup>+</sup> T cells from EC-1, -2, and -11 exhibited significant resistance to the YU2, JRFL, and ADA R5-tropic envelopes, compared to normal controls (again, EC-3 was excluded from this analysis because of limited sample availability) (Fig. 1E). In contrast, these cells were fully susceptible to viruses containing the HXB2-, NL4.3-, and LAI X4-tropic envelopes as well as VSV-G (Fig. 1E).

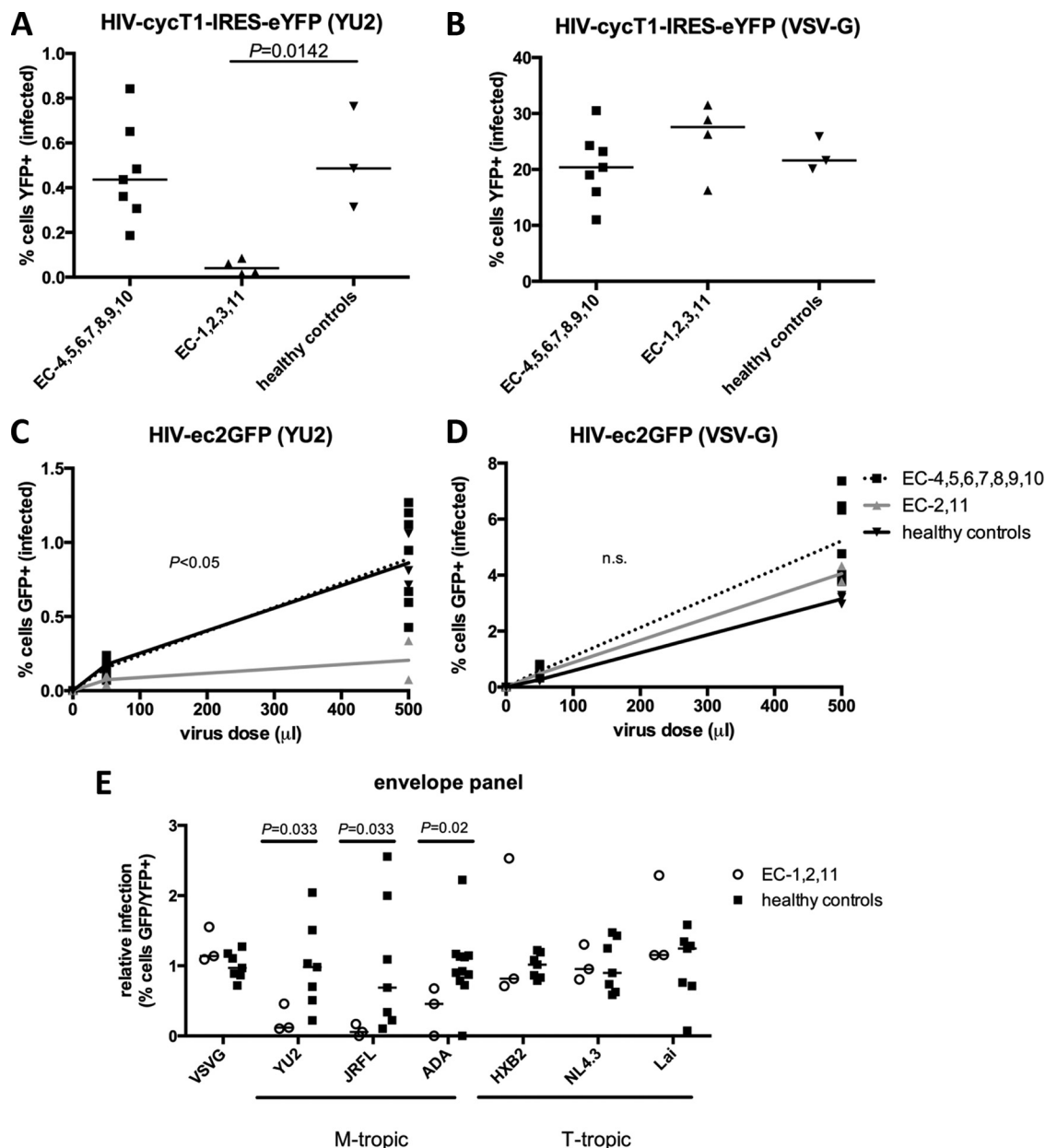
**Cellular resistance to R5-tropic HIV is not associated with altered CCR5 levels.** R5-tropic HIV envelopes mediate entry via the CCR5 coreceptor, whereas X4-tropic HIVs utilize the CXCR4 coreceptor. To determine if altered CCR5 levels explained this

TABLE 1 Characteristics of the EC cohort<sup>a</sup>

Patient	Ethnicity	Race	Sex	Age (yr)	No. of CD4 cells	% CD4 cells	Viral load(s) (date [mo/yr])	ART (yr)	Yr of diagnosis	Risk factor(s)	Comorbidity(ies)	HLA type
EC-1	H	W	M	42	429	31	<48 (2010–2012), 2,290 (2013), <20 (2014)	Efavirenz, emtricitabine, and tenofovir (2014)	2010	Heterosex	Hyperchol, GERD	B*15/B*44
EC-2	NH	B	F	50	1272	62	<50 (6/1999), ND (2000, 2001, 2002, 2003, 2006, 2007, 2008, 2009, 2010, 2012), <20 (10/2013)		1997		splenectomy, HCV, DM, HTN	B*49/B*57:03:01
EC-3	NH	W	M	47	354	43	ND (2001, 2002, 2003, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014)	Efavirenz, emtricitabine, and tenofovir (2010–2014)	1989	IVDU	Rectal cancer, LTBI	B*13/B*51
EC-4	NH	B	M	65	536	36	ND (1996–2014)		1989		CKD, HCV, LTBI, AKA, DM, HTN, Dyslipid	B*44/B*57:03:01
EC-5	NH	B	M	49	1,204	46	ND (2010), ND (2/2012, 5/2012), <20 (7/2012), <20 (3/2013), <20 (7/2013), ND (10/2013), ND (1/2014), 31 (3/2014)		1988	IVDU	HCV, DM, Dyslipid	B*51/B*57:03:01
EC-6	NH	B	F	55	846	43	ND (2010), <20 (7/2012), <20 (3/2013), <20 (7/2013), ND (10/2013), ND (1/2014), 31 (3/2014)		2010	Heterosex	Dyslipid	B*14/B*27
EC-7	NH	B	F	46	1,051	39	ND (1998, 1999, 2001, 2005, 2006, 2007), <48 (2008), ND (1/2009), <48 (9/2009), 57 (6/2010), <48 (11/2010), <20 (8/2011), <20 (2/2012), ND (8/2012), 57 (4/2013), 24 (8/2013)		1988		HCV	B*35/B*57:03:01
EC-8	NH	B	F	52	1,779	54	ND (1999, 2000, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2011, 2012, 2013)		1990		HTN	B*27/B*57:03:01
EC-9	H	W	F	53	907	50	ND (6/2008), ND (6/2010), <20 (2014)		1995	Heterosex	HTN, asthma	B*07/B*57:03:01
EC-10	NH	W	M	57	932	31	ND (1992–2010), <48 (2010, 2011), 77 (2012), 128 (2013)		1992	MSM	HTN, CAD MI, GERD, asthma, Hyperchol	B*07/B*57:01:01
EC-11	NH	B	M	60	308	27	<48 (2010), 102 (2011), 49 (2012), <48 (2013)		2010	IVDU, heterosex	GIST, partial gastrectomy, DM, HTN, Dyslipid	B*07/B*57:03:01

<sup>a</sup> AKA, above-the-knee amputation; B, black; CAD, coronary artery disease; CKD, chronic kidney disease; DM, diabetes mellitus; dyslipid, dyslipidemia; GERD, gastroesophageal reflux disease; GIST, gastrointestinal stromal tumor; H, Hispanic; HCV, hepatitis C virus; heterosex, heterosexual contact; HTN, hypertension; Hyperchol, hypercholesterolemia; IVDU, intravenous drug use; LTBI, latent tuberculosis; MI, myocardial infarction; MSM, men who have sex with men; ND, nondetectable; NH, not Hispanic; W, white.

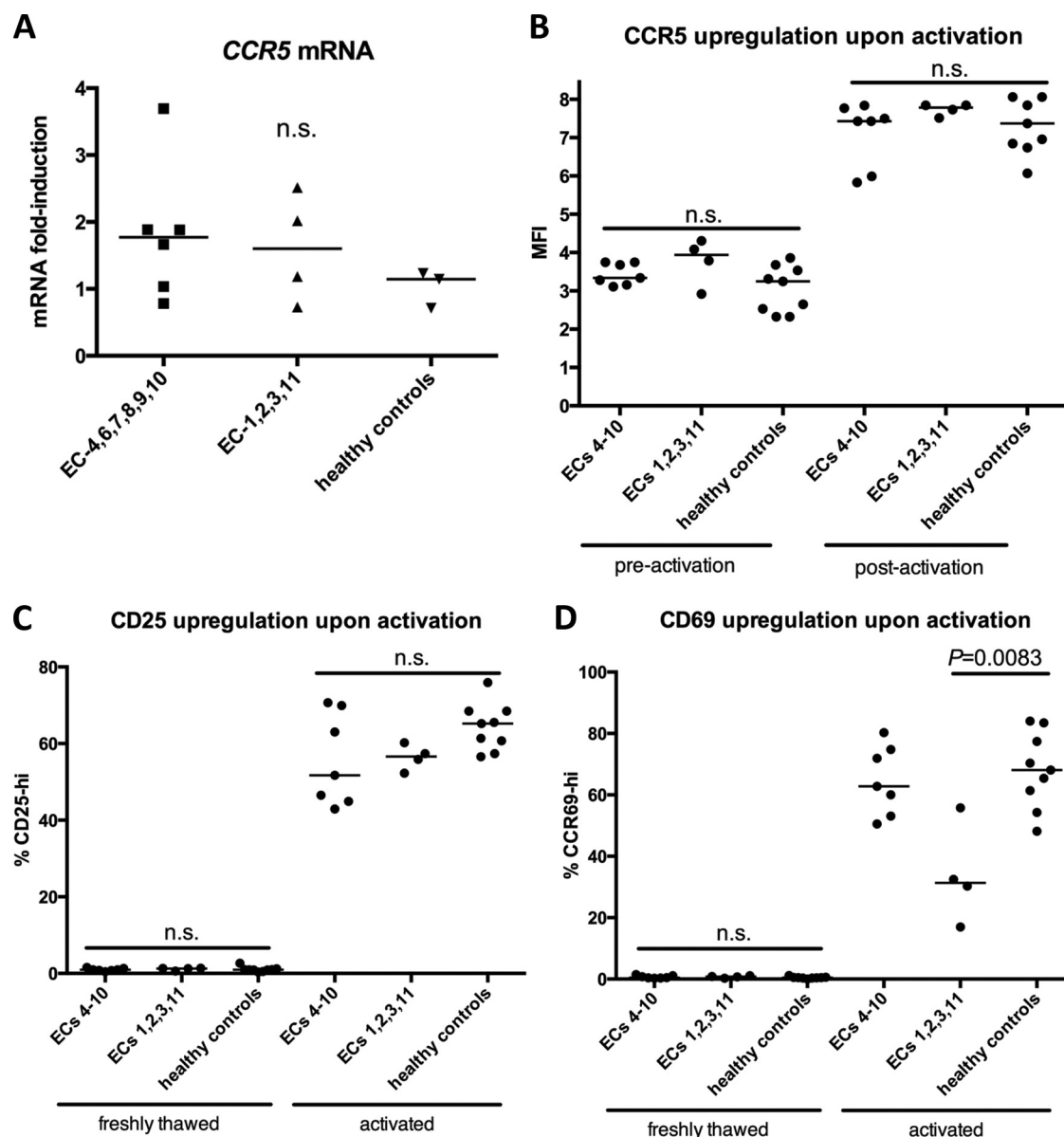




**FIG 1** A subset of ECs shows CD4<sup>+</sup> T cell resistance to R5-tropic HIV. (A and B) CD4<sup>+</sup> T cells from the ECs and healthy controls were activated for 3 days with anti-CD3 and anti-CD28.2 in the presence of IL-2. Cells were then infected with replication-defective HIV-cycT1-internal ribosome entry site (IRES)-eYFP pseudotyped with YU2 (R5 tropic) (A) or VSV-G (pantropic) (B). (C and D) A dose response analysis was performed with replication-defective HIV-ec2GFP pseudotyped with the YU-2 envelope (C) or VSV-G (D). Panels A to D show results of one representative experiment with 3 healthy controls. Data were analyzed by using the Kruskal-Wallis test and Dunn's multiple-comparison test. (E) Replication-defective HIV-cycT1-IRES-eYFP or HIV-ec2GFP pseudotyped with a panel of envelopes, including VSV-G, YU2, JRFL, and ADA (R5) and HXB2, NL4.3, and Lai (X4). Shown are pooled results from three experimental repeats with 11 healthy control samples for the ADA envelope and 7 healthy control samples for the other envelopes. Data were analyzed separately for each envelope with the Mann-Whitney test. Graphs show individual values and medians. n.s., not significant.

phenotype, we harvested RNA from CD4<sup>+</sup> T cells after activation and measured CCR5 levels by reverse transcription-quantitative PCR (qRT-PCR). We observed similar CCR5 mRNA levels in all subjects (Fig. 2A). Additionally, we quantified CCR5 surface protein levels on CD4<sup>+</sup> T cells by flow cytometry. We observed a substantial upregulation of CCR5 upon activation of CD4<sup>+</sup> T cells, and this occurred to a similar degree in all subjects (Fig. 2B). Genotyping of CCR5, via qPCR, demonstrated that none of the

ECs in this cohort possessed the  $\Delta 32$  CCR5 mutation, which prevents R5-tropic HIV entry (48). Additionally, whole-exome sequencing demonstrated that none of these subjects had mutations in their CCR5 or CD4 exonic sequences. Hence, altered CCR5 coding sequences or levels of expression did not account for resistance to R5-tropic virus. Upon CD4<sup>+</sup> T cell activation, CD25 was upregulated to a similar degree in all of the ECs and healthy controls, suggesting a comparable activation status (Fig. 2C). How-



**FIG 2** CCR5 mRNA levels and CCR5 and CD25 protein levels are not altered in  $CD4^+$  T cells from resistant ECs, while CD69 levels are reduced in resistant ECs. (A) mRNA levels of *CCR5* in activated  $CD4^+$  T cells relative to the *EF1a* control (results from one representative experimental repeat with 3 healthy controls). (B to D) Cell surface levels of CCR5 (B), CD25 (C), and CD69 (D), as measured by flow cytometry, in freshly thawed and activated  $CD4^+$  T cells (results from two pooled experiments with 9 healthy controls). Data were analyzed with the Kruskal-Wallis test and Dunn's multiple-comparison test. Graphs show individual values and medians.

ever, CD69 upregulation was slightly reduced in resistant ECs 1, 2, 3, and 11 (Fig. 2D).

**Transcriptional signatures associated with the cellular R5-tropic HIV resistance phenotype.** We investigated whether specific transcriptional signatures were associated with the  $CD4^+$  T cell resistance phenotype. We activated  $CD4^+$  T cells from two of the resistant ECs (EC-2 and EC-11) and three healthy controls, prepared RNA, and performed exploratory RNA-Seq transcriptome analyses (EC-1 and EC-3 were again excluded from this analysis because of limited sample availability). The levels of 17 genes were statistically significantly different between the resistant ECs and healthy controls (Table 2). Of the genes on this list, we

were intrigued by the presence of *CCL3* and *CCL3L1*, both of which encode MIP-1 $\alpha$ . MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES are natural ligands of CCR5 that inhibit R5-tropic HIV infection by steric hindrance of virus binding (49–52). The *CCL3* and *CCL3L1* genes were both upregulated in EC-11 versus healthy controls. In contrast, EC-2 had only slightly elevated levels of *CCL3* and levels of *CCL3L1* similar to those in healthy controls (Table 2).

**Cellular resistance to R5-tropic HIV is associated with increased levels of MIP-1 $\alpha$  and/or MIP-1 $\beta$  in EC-1, -3, and -11.** To determine whether the secreted amounts of MIP-1 $\alpha$  and the related chemokines MIP-1 $\beta$  and RANTES were elevated, we harvested supernatants from activated  $CD4^+$  T cells immediately

TABLE 2 Transcriptome analysis of activated CD4<sup>+</sup> T cells from EC-2 and EC-11 versus healthy controls<sup>a</sup>

Gene	Transcriptome expression value (no. of fragments/kb of exon/million fragments mapped)						Fold change	P value
	EC-2	EC-11	HC-1	HC-2	HC-3	EC mean		
<i>IFIT2</i>	1.94	21.55	0.68	1.75	3.35	11.78	6.09	2.17E−06
<i>IFIT3</i>	3.17	31.30	1.49	1.92	6.42	17.24	5.24	1.23E−05
<i>LYZ</i>	21.51	21.49	7.77	3.16	5.01	22.18	4.04	6.73E−05
<i>IFNG</i>	495.03	222.40	180.08	86.80	54.94	357.77	3.33	1.03E−06
<i>CCL3</i>	93.34	296.99	60.19	47.49	65.60	196.01	3.35	8.93E−06
<i>CCL3L1</i>	1.52	38.95	1.02	0.35	3.33	20.26	12.75	2.79E−06
<i>IL1B</i>	37.06	50.56	10.20	6.02	9.63	43.98	5.10	1.17E−07
<i>CLDN1</i>	0.70	0.52	1.41	10.52	0.30	0.60	0.15	6.87E−05
<i>IL-8</i>	46.05	126.72	22.54	10.24	22.57	83.91	4.86	7.74E−09
<i>CXCL5</i>	0.22	9.00	0.80	0.35	0.63	4.60	8.01	3.06E−05
<i>IL-2</i>	0.69	9.10	0.12	0.41	0.18	4.89	0.24	3.15E−05
<i>IL-9</i>	178.61	60.90	22.08	9.42	3.15	131.12	12.31	4.15E−10
<i>LTA</i>	231.21	324.48	62.05	105.74	76.26	272.36	3.40	1.78E−07
<i>TNF</i>	19.55	27.70	5.15	5.20	8.23	22.72	3.79	5.58E−05
<i>IL17A</i>	5.50	49.91	3.23	3.80	6.26	27.58	4.39	4.15E−07
<i>IL17F</i>	243.39	777.42	133.68	74.58	44.55	513.38	5.97	9.06E−12
<i>IDO1</i>	19.03	31.55	10.72	3.24	4.41	25.27	4.09	1.27E−05

<sup>a</sup> Transcriptome expression values (fragments per kilobase of exon per million fragments mapped) for EC-2, EC-11, and three healthy controls (HCs) (HC-1, HC-2, and HC-3) are shown. For the 17 genes shown, expression values were significantly different between ECs and healthy controls. Please note that this table omits a duplicate entry for *CCL3L1*; two processed pseudogenes, *PSPHP1* and *RP11-10B2.1.1*; and two transcripts with no database entry, which were also significant. *P* values were calculated from the difference in the distribution of observed log fold changes (i.e., Student's *t* test on the log<sub>2</sub> fold change) by using Cufflinks.

prior to the infection assay and measured chemokine levels via an ELISA. T cells from EC-3 and EC-11 exhibited a significant increase in MIP-1α production relative to healthy controls (Fig. 3A), whereas levels for EC-1 and EC-2 were similar to those for healthy controls. The level of MIP-1β production was elevated in EC-1, EC-3, and EC-11 relative to healthy controls (Fig. 3B), whereas EC-2 levels were similar to those in healthy controls. RANTES production did not show a significant differences between the samples (Fig. 3C). Additionally, we measured MIP-1α levels in serum isolated from these patients. In contrast to the results obtained for activated CD4<sup>+</sup> T cell media, MIP-1α levels were similar in the sera of both resistant ECs and healthy controls (Fig. 3D).

**CCL3-like and CCL4-like gene copy numbers are elevated in EC-1, EC-2, and EC-3, and the *STAT-1* mRNA expression level is elevated in EC-11.** The *CCL* genes encoding MIP-1α, MIP-1β, and RANTES lie in a cluster at chromosome 17q12 (53). The original genes, *CCL3* and *CCL4*, are each present as a single copy and encode MIP-1α and MIP-1β, respectively. However, segmental duplication of this region has resulted in additional genes that exhibit copy number variation (CNV) (47). This CNV locus includes the full-length *CCL3-like* (*CCL3L*) genes *CCL3L1* and *CCL3L3* as well as a truncated gene, *CCL3L2*. These genes exhibit a high degree of homology to each other and the *CCL3*, with *CCL3L1* being identical to *CCL3L3* at the nucleotide level. Likewise, there are multiple *CCL4-like* (*CCL4L*) genes in this region, including *CCL4L1* and *CCL4L2*, which are identical to each other at the amino acid level and highly homologous to *CCL4*. Moreover, this CNV region exhibits a complex architecture, with variations in breakpoints and CNVs inserted into larger CNVs (47).

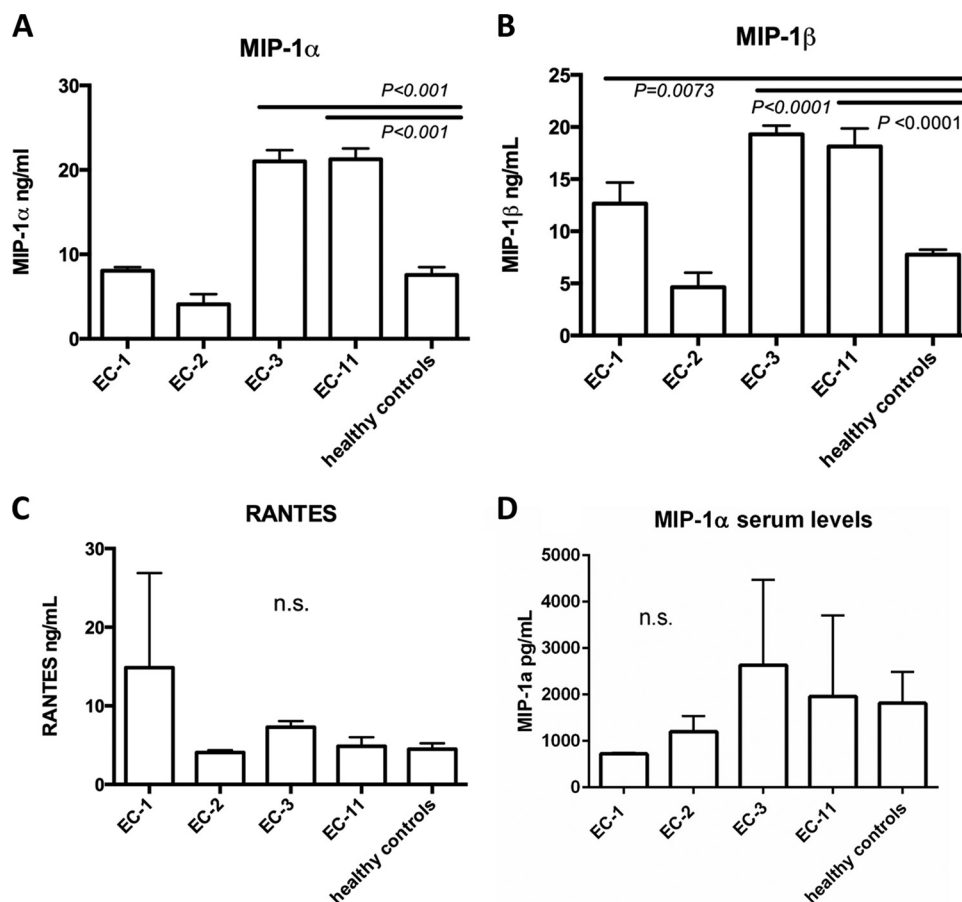
The relative *CCL3L* gene copy number varies among different ethnic and racial backgrounds. The *CCL-like* gene copy number regulates chemokine production levels (45, 46). Some studies suggest that individuals with a high gene copy number (*n*) in relation

to their racial background are less likely to seroconvert and have a lower rate of HIV progression to AIDS if seropositive (46).

*CCL3-like* and *CCL4-like* gene copy numbers were therefore quantified by qPCR. Both EC-1 and EC-3 exhibited high relative copy numbers of *CCL3-like* genes (*n* = 17.0 and 11.8, respectively) (Fig. 4A) and *CCL4-like* genes (*n* = 8.4 and 8.1, respectively) (Fig. 4B) for individuals of European descent. It is therefore likely that the higher relative copy numbers of *CCL3-like* genes and *CCL4-like* genes explain the increased levels of production of MIP-1α in EC-3 and MIP-1β in both individuals. Additionally, EC-2 exhibited a somewhat elevated relative gene copy number for an individual of African descent (*CCL3-like* genes, *n* = 13.3 [Fig. 4A]; *CCL4-like* genes, *n* = 6.1 [Fig. 4B]), although we detected a normal level of chemokine production by ELISA and transcriptome analyses (Fig. 3 and Table 2). In contrast, EC-11 exhibited an intermediate relative gene copy number for an individual of African ancestry (*CCL3-like* genes, *n* = 6.7 [Fig. 4A]; *CCL4-like* genes, *n* = 3.2 [Fig. 4B]). It is therefore unlikely that the gene copy number is responsible for the increased levels of chemokines in EC-11. Whole-exome sequencing demonstrated that none of the patients in our cohort had mutations in *CCL3* or *CCL4* exonic sequences.

In order to further explore the mechanism of the increase in MIP levels in EC-11, we utilized qPCR to quantify the levels of three transcription factors that bind to the promoters of the *CCL3*, *CCL3-like*, *CCL4*, and *CCL4-like* genes. *STAT-1* mRNA levels were elevated in EC-11 T cells versus healthy control T cells (Fig. 4C). In contrast, *RUNX1* and *C/EBPα* mRNA levels were similar between EC-11 and control T cells (data not shown).

**MIP-1α and MIP-1β produced by EC-3 and EC-11 CD4<sup>+</sup> T cells are necessary and sufficient to confer the R5-tropic HIV cellular resistance phenotype in a medium transfer experiment.** We employed a medium transfer experiment to determine whether the high levels of MIP-1α and MIP-1β were responsible



**FIG 3** Increased production of CCR5 ligands by EC-3 and EC-11 CD4<sup>+</sup> T cells. (A to C) CD4<sup>+</sup> T cells from EC-2, EC-3, EC-11, and healthy controls were activated for 3 days, and levels of chemokines in the culture supernatant, including MIP-1 $\alpha$  (A), MIP-1 $\beta$  (B), and RANTES (C), were measured by an ELISA. Pooled results from three experimental repeats with 11 healthy control samples are shown. (D) Serum was obtained from EC-1, EC-2, EC-3, EC-11, and healthy controls, and the MIP-1 $\alpha$  level was measured by an ELISA. Pooled results from two experimental repeats with 9 healthy control samples are shown. Data were analyzed by one-way analysis of variance and Bonferroni's multiple-comparison test. Graphs show mean values and standard errors of the means.

for the observed T cell-intrinsic resistance of EC-3 and EC-11. We treated activated CD4<sup>+</sup> T cells from a healthy donor with cell culture supernatants from EC-3, EC-11, or healthy control CD4<sup>+</sup> T cells and then infected these cells with YU2-pseudotyped HIV. EC-3 and EC-11 supernatants suppressed YU2-pseudotyped HIV infection relative to healthy control supernatants (Fig. 5A). Furthermore, the addition of anti-MIP-1 $\alpha$  and anti-MIP-1 $\beta$  neutralizing antibodies reversed this effect (Fig. 5A). These data suggest that a secreted factor(s) from EC-3 and EC-11 T cells confers cellular resistance to R5-tropic HIV and that MIP-1 $\alpha$  and MIP-1 $\beta$  are necessary for this effect. In contrast, supernatants from EC-3 and EC-11 T cells did not alter infection by HXB2-pseudotyped HIV (Fig. 5B).

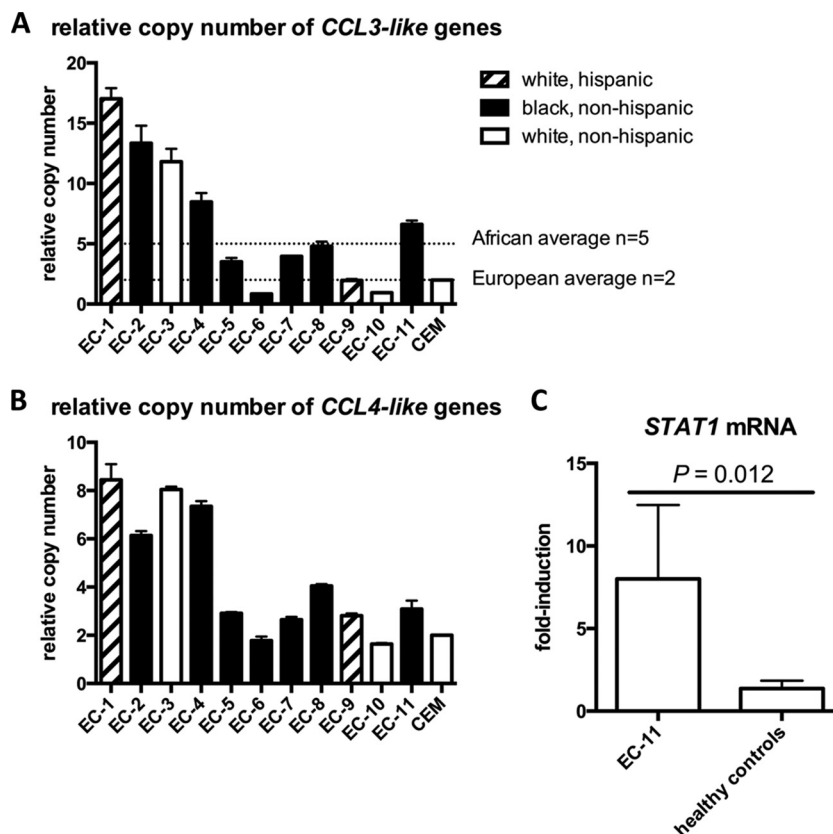
In a separate experiment, we treated CD4<sup>+</sup> T cells from healthy controls with culture supernatants from cells transfected with pCCL3L1 (encoding MIP-1 $\alpha$ ), pCCL4 (encoding MIP-1 $\beta$ ), a control plasmid encoding  $\beta$ -arrestin, or complete RPMI. These cells were then infected with YU2-pseudotyped virus. Supernatants containing MIP-1 $\alpha$  or MIP-1 $\beta$  suppressed YU2-pseudotyped HIV infection relative to that of the  $\beta$ -arrestin control (Fig. 5C). The addition of the appropriate neutralizing antibody reversed this effect (Fig. 5C). These data indicate that MIP-1 $\alpha$  and MIP-1 $\beta$  are indeed sufficient to inhibit R5-tropic HIV infection.

## DISCUSSION

This study demonstrates a novel HIV cellular resistance phenotype in a subset of ECs: CD4<sup>+</sup> T cells were intrinsically resistant to R5-tropic HIV but fully susceptible to X4-tropic and VSV-G-pseudotyped HIV for 4 of 11 patients in our cohort (EC-1, -2, -3, and -11). The remaining patients in the cohort likely accomplish HIV control via other mechanisms that may be CD4<sup>+</sup> T cell extrinsic. These and previous studies support the notion that ECs are a diverse group of patients who accomplish HIV control via multiple distinct and unknown mechanisms.

Previous studies investigated whether ECs harbor CD4<sup>+</sup> T cells that are intrinsically resistant to HIV infection but obtained mixed results (24–34). We speculate that these conflicting results are a consequence of the different experimental conditions among these studies. Viral infection rates may be influenced by the CD4<sup>+</sup> T cell activation protocol, the physiological state of the cells, the viral strains used, viral titers, cell density, the frequency of medium change, and the use of spinoculation or lack thereof. It is possible that the specific experimental conditions used in our study are critical to observe the phenotype of R5-tropic resistance in a subset of ECs. Alternatively, the patient characteristics of our cohort may be unique. Although it would be of interest to deter-





**FIG 4** High relative copy numbers of *CCL3-like* and *CCL4-like* genes in EC-1, EC-2, and EC-3 and elevated *STAT-1* mRNA levels in EC-11. (A and B) Relative copy numbers of *CCL3-like* (A) and *CCL4-like* (B) genes in ECs relative to the CEM cell line ( $n = 2$ ). The dashed line indicates the average *CCL3L1* copy number for individuals of African and European descent. The bar pattern indicates the ethnicity of each individual. Graphs show pooled results from two qPCR assays, each run in duplicate. Data were analyzed by one-way analysis of variance and Bonferroni's multiple-comparison test. (C) *STAT-1* mRNA levels in EC-11 versus healthy controls relative to the *EF1a* mRNA control. Graphs show pooled results from two experimental repeats with 8 healthy control samples. Data were analyzed with a  $t$  test. Graphs show mean values and standard errors of the means.

mine HIV tropism in these ECs, the low levels of plasma viremia make it extremely challenging to do so.

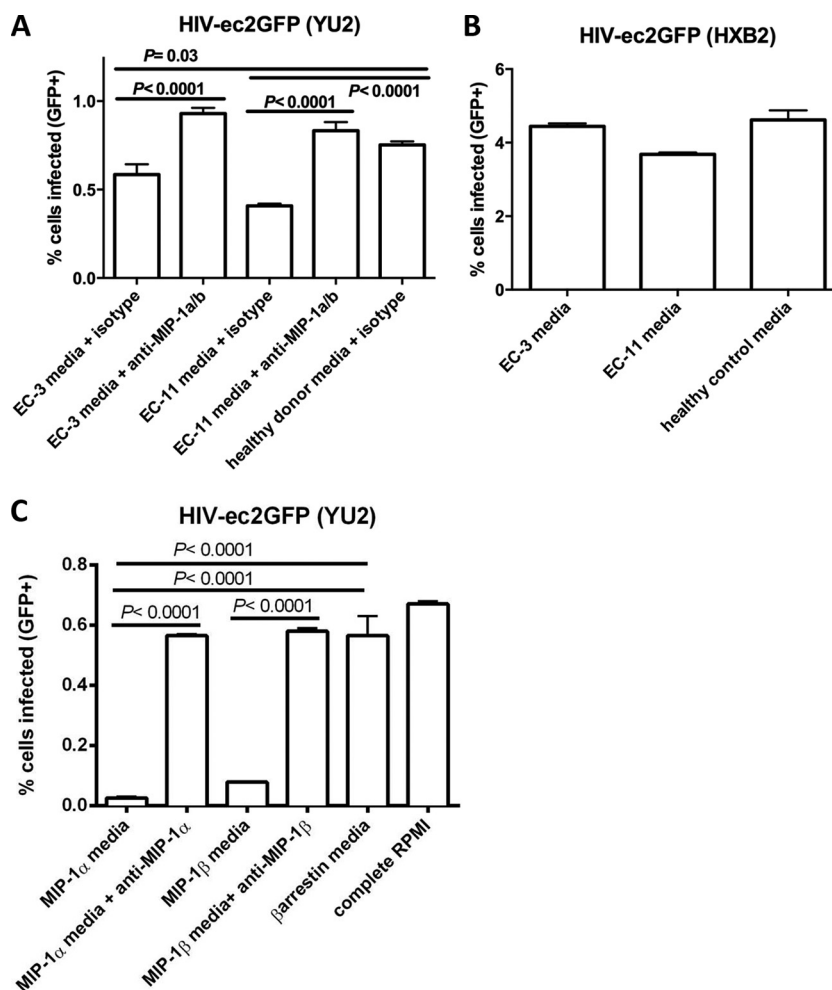
For three patients in our cohort, EC-1, EC-3, and EC-11, we determined that increased levels of MIP-1 $\alpha$  and/or MIP-1 $\beta$  secretion were associated with cellular resistance. Activated CD4<sup>+</sup> T cells from these patients produced significantly more MIP-1 $\alpha$  (EC-3 and EC-11) and more MIP-1 $\beta$  (EC-1, EC-3, and EC-11) than did those from healthy controls. The level of RANTES production was not elevated in these patients. We did not observe an elevation in chemokine levels in the sera of these patients. It is likely that CD4<sup>+</sup> T cells must be activated to induce chemokine production. Alternatively, it is possible that even unactivated CD4<sup>+</sup> T cells produce more chemokines in these patients, but this is not sufficient to elevate serum levels. Even in the absence of elevated serum chemokine levels, MIP-1 $\alpha$  locally produced by activated CD4<sup>+</sup> T cells could bind cell surface CCR5 in an autocrine or paracrine fashion, inhibiting the entry of R5-tropic HIV.

High relative copy numbers of *CCL3-like* and *CCL4-like* genes were detected in EC-1 and EC-3 and likely account for the increased chemokine production that we observed in CD4<sup>+</sup> T cells from these patients. Interestingly, the relative copy numbers of *CCL3-like* and *CCL4-like* genes were also somewhat elevated in EC-2. However, this individual displayed normal *CCL3L1* gene expression levels and normal MIP-1 $\alpha$ / $\beta$  levels in stimulated T cell

culture supernatants. It remains plausible that an elevated *CCL* copy number is somehow mechanistically responsible for cellular resistance in EC-2 despite not exhibiting an obvious elevation at the RNA or protein level in CD4<sup>+</sup> T cells. In contrast, EC-11 had intermediate relative copy numbers of *CCL3-like* and *CCL4-like* genes for an individual of African ancestry. Hence, the copy number does not explain the elevated levels of chemokine production that we observed for this individual. EC-11 exhibited elevated mRNA levels of *STAT-1*, a transcription factor with a binding site in the *CCL* gene promoters. Elevated *STAT-1* levels may thus play a role in cellular resistance in EC-11. However, the mechanism for the elevation of *STAT-1* mRNA levels in this individual remains to be fully explored.

In a medium transfer experiment, secreted products from EC-3 and EC-11 CD4<sup>+</sup> T cells were sufficient to confer cellular resistance to R5-tropic HIV. MIP-1 $\alpha$  and MIP-1 $\beta$  were necessary for this effect, since chemokine-neutralizing antibodies fully reversed the phenotype. Additionally, transfected medium containing the MIP-1 $\alpha$  or MIP-1 $\beta$  chemokine suppressed R5-tropic HIV infection, indicating that the chemokines themselves were also sufficient for these effects, consistent with data from previous reports (30, 54).

Previous studies obtained mixed results regarding the association between the *CCL3L1* and *CCL4L1* copy numbers and HIV/



**FIG 5** MIP-1 $\alpha$  and MIP-1 $\beta$  are necessary and sufficient to confer R5-tropic HIV resistance in a medium transfer experiment. (A and B) CD4<sup>+</sup> T cells from healthy donors were treated with the activated CD4<sup>+</sup> T cell culture supernatant from EC-3 or EC-11 in the presence or absence of anti-MIP-1 $\alpha$  and -MIP-1 $\beta$  neutralizing antibodies or with the activated CD4<sup>+</sup> T cell culture supernatant from a healthy control prior to the addition of HIV particles pseudotyped with YU2 (A) or HXB2 (B). (C) CD4<sup>+</sup> T cells from healthy controls treated with the cell culture supernatant from 293T cells transfected with either pCCL3L1, encoding MIP-1 $\alpha$ , or pCCL4, encoding MIP-1 $\beta$ , in the presence or absence of the appropriate neutralizing antibodies; medium from cells transfected with a  $\beta$ -arrestin-encoding plasmid; or complete RPMI, prior to the addition of YU2-pseudotyped HIV particles. Graphs show results from one representative experimental repeat, run in duplicate. Data were analyzed by one-way analysis of variance and Bonferroni's multiple-comparison test. Graphs show mean values and standard errors of the means.

AIDS susceptibility and disease progression (reviewed by Hollox and Hoh [55]). For example, some reports demonstrated an association between high *CCL3L1* copy numbers and a reduced prevalence of HIV seropositivity, slower progression to AIDS, and improved recovery of CD4<sup>+</sup> T cells (45, 56–58), whereas other studies and follow-up correspondences failed to detect an association even within the same data set (59–61). Some researchers have suggested that the *CCL3L1* copy number estimation method used in those association studies could be flawed, for example, due to DNA degradation, inconsistent quality among DNA samples, the use of inappropriate DNA amounts in the real-time PCR assay, inappropriate comparison of threshold cycle ( $C_T$ ) values from samples run in different real-time PCR experimental repeats, or inappropriate rounding of copy numbers to the nearest integer value. Of note, every DNA sample used in our study was isolated by the same investigator, using the same purification method. The DNA obtained was found to be of high quality, with a 260/280-nm

light absorbance ratio of  $\sim 1.8$  and no obvious degradation, as evidenced by agarose gel electrophoresis of the samples. The amount of DNA used for the real-time PCR assay was 6.6 ng for every sample, and all samples were included in every experimental replicate, with consistent values being observed between replicates. A serial dilution of the *CCL3L1* plasmid DNA and genomic DNA suggested that the  $C_T$  of the samples was within the range in which the  $2^{\Delta\Delta C_T}$  method yielded an appropriate estimation of the gene copy number. We did not round our copy number values to an integer but instead reported them to two or three significant digits. Finally, previous reports criticized the fact that multiple reports that found a positive association used duplicated patient cohorts. In contrast, the patient cohort in this report is unique and was not used in any previous studies.

Protective HLA alleles were previously implicated as a determining factor in HIV control versus progression (10). Most notably, the B\*57 alleles are associated with HIV control. Seven of 11

patients in our cohort possessed B\*57 alleles (EC-2, EC-4, EC-5, EC7, EC-8, EC-9, and EC-11), including two of the elite controllers who exhibited the pattern of R5-tropic cellular resistance described in this report (EC-2 and EC-11). It is plausible that CD4<sup>+</sup> T cell resistance synergizes with an HLA-based mechanism (i.e., enhanced CD8<sup>+</sup> T cell response to HIV antigens) to control HIV replication in these patients. We also observed reduced CD69 up-regulation upon CD4<sup>+</sup> T cell activation in the resistant ECs versus healthy controls. The significance of this finding is unclear.

In summary, the factors contributing to the natural control of HIV are likely multifactorial. Increased production of MIP-1 $\alpha$  and MIP-1 $\beta$  can now be added as a mechanism that drives cellular resistance to R5-tropic virus in some of these individuals, likely contributing to their EC phenotype. Future studies will be required to determine precisely how the levels of these chemokines are elevated in EC T cells and their relative importance among all ECs and LTNP. While limited by a small sample size, our findings support the continued investigation of coreceptors as a target for drug development and functional eradication of HIV. Whether the expression levels of the natural ligands for CCR5 or CXCR4 can be increased for therapeutic benefit with minimal risk remains to be explored.

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